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- Published:**
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WO 01/72296 A1

(54) Title: METHOD OF INHIBITING THE EXPRESSION OF INFLAMMATORY CYTOKINES AND CHEMOKINES

(57) Abstract: A method of inhibiting the expression of inflammatory cytokines and chemokines in an animal or man, is disclosed. The method comprises administration to said animal or man of at least one type of xanthophyll, e.g. astaxanthin, in an amount inhibiting the expression of inflammatory cytokines and chemokines in said animal or man. Use of at least one type of xanthophyll, such as astaxanthin, for the preparation of a medicament for the prophylactic and/or therapeutic inhibition of the expression of inflammatory cytokines and chemokines in an animal or man, is described. Further, a commercial package containing a medicament comprising at least one type of xanthophyll, e.g. astaxanthin, and written and/or data carrier instructions for administration to an animal or man of the medicament for the prophylactic and/or therapeutic inhibition of the expression of inflammatory cytokines and chemokines, is disclosed.

Method of inhibiting the expression of inflammatory cytokines and chemokines

The present invention relates to a method of inhibiting the expression of inflammatory cytokines and chemokines in an animal or man, and to the use of a xanthophyll, e.g. astaxanthin, for the preparation of a medicament for the prophylactic and/or therapeutic inhibition of the expression of inflammatory cytokines and chemokines.

Background

The nuclear factor- κ B (NF- κ B) is a conditionally regulated transcription factor that plays a key role in the expression of a variety of genes involved in inflammation, cell survival, apoptosis, cell differentiation and cancer. It was first identified as a regulator of κ light chain expression in murine B-lymphocytes, but has now been shown to be expressed ubiquitously and to be a master regulator of several important processes. The NF- κ B family consists of structurally related proteins of the Rel family, including p50, p52, p65/RelA, c-Rel and RelB (reviewed in Rothwarf and Karin, 1999). In unstimulated cells, NF- κ B is bound to the inhibitor protein I κ B, which masks the nuclear localisation signal of NF- κ B and retains it in the cytoplasm. Activation of the cell with various stimuli initiates signalling pathways involving activation of a whole series of protein kinases. This results in phosphorylation of I κ B, targeting the protein for degradation (Rothwarf and Karin, 1999). As a result, the I κ B/NF- κ B complex dissociates, NF- κ B translocates to the nucleus and binds to its cognate sites. Nuclear translocation of NF- κ B is activated by various stimuli, including the inflammatory cytokines TNF- α and IL-1, UV-irradiation, mitogens, viruses, bacteria, double stranded DNA, ionizing radiation and hydrogen peroxide, in accordance with the important role played by NF- κ B in various tissues (Rothwarf and Karin, 1999).

The functional importance of NF- κ B in acute and chronic inflammation is based on its ability to regulate the promoters of a variety of genes. The products of such genes are e.g. cytokines, adhesion molecules and acute phase proteins, which are critical for inflammatory processes (Baeuerle *et al.*, 1995, Shakov *et al.*, 1990, Libermann *et al.*, 1990). These findings are further underlined by the demonstration that mice containing targeted disruptions of the NF- κ B subunits p50, RelB and c-Rel are compromised in various aspects of immune function and inflammatory processes (Sha *et al.*, 1995; Weih *et al.*, 1995, Köntgen *et al.*, 1995). Moreover, elevated levels of p65 have been observed in patients with rheumatoid arthritis and Inflammatory Bowel Disease (IBD). A role for NF- κ B in inflammation was further established in a recent study, demonstrating that local administration of an antisense

oligonucleotide targeted against the translational start site of NF- κ B p65 abrogates established intestinal inflammation in mice (Neurath *et al.*, 1996).

During the last years, it has become evident that redox regulation is an important mechanism that regulates conditional gene expression. Several transcription factors have been shown to be redox-regulated, including NF- κ B. One common step in the activation mechanisms that lead to NF- κ B translocation has been suggested to involve reactive oxygen species, based on the finding that NF- κ B activation can be inhibited by a series of antioxidants (reviewed in Pitette *et al.*, 1997). However, little is known about the pathways that activate and control NF- κ B e.g. during oxidative stress.

Due to the involvement of the transcription factor NF- κ B in inflammatory processes it could be possible to inhibit the expression of inflammatory cytokines and chemokines by affecting the function of the NF- κ B in an animal or human.

Description of the invention

The present invention provides a method to inhibit the expression of inflammatory cytokines and chemokines in an animal or man.

The method of the invention comprises administration to an animal or man of at least one type of xanthophyll in an amount inhibiting the expression of inflammatory cytokines and chemokines in said animal or man.

Examples of inflammatory cytokines are TNF- α and IL-1, and examples of chemokines are MIP-2, CXC 5 and CXC 6.

The daily doses of the xanthophyll for inhibiting the expression of inflammatory cytokines and chemokines will normally be in the range of 0.01 to 50 mg per kg body weight of an animal or human, but the actual dose will be decided based on the recommendations of the manufacturer of the medicament comprising the xanthophyll.

In an embodiment of the method of the invention the type of xanthophyll is astaxanthin. The astaxanthin may be selected from the group consisting of astaxanthin from a natural source, such as a culture of the alga *Haematococcus sp.*, synthetic astaxanthin and mixtures thereof.

Astaxanthin from other natural sources than algae, such as from fungi and crustaceans, and other xanthophylls as well, are expected to be similarly useful for the purposes of the invention. An advantage of using astaxanthin from algae may be that the astaxanthin exists in a form esterified with fatty acids, which esterified astaxanthin thereby is more stable during handling and storage than free astaxanthin.

Another aspect of the invention is directed to the use of at least one type of xanthophyll for the preparation of a medicament for the prophylactic and/or therapeutic inhibition of the expression of inflammatory cytokines and chemokines in an animal or man.

In a preferred embodiment the type of xanthophyll is astaxanthin. The
5 astaxanthin may be selected from the group consisting of astaxanthin from a natural source, such as a culture of the alga *Haematococcus sp.*, synthetic astaxanthin and mixtures thereof.

Yet another aspect of the invention is directed to a commercial package containing a medicament comprising at least one type of xanthophyll and written and/or data carrier instructions for administration to an animal or man of the medicament for the
10 prophylactic and/or therapeutic inhibition of the expression of inflammatory cytokines and chemokines.

The medicament preferably comprises astaxanthin, selected from the group consisting of astaxanthin from a natural source, such as a culture of the alga *Haematococcus sp.*, synthetic astaxanthin and mixtures thereof.

15 The commercial package of the invention may additionally contain a water soluble antioxidant, such as glutathione and/or ascorbic acid (vitamin C) and/or a fat soluble antioxidant other than the xanthophyll, such as tocopherol (vitamin E).

The commercial package and/or the medicament comprised by the present invention may comprise additional ingredients which are pharmacologically acceptable
20 inactive or active in prophylactic and/or therapeutic use, such as excipients and flavouring agents.

Description of the drawing

Fig 1 is a diagram which shows that Astaxanthin inhibits the activation of NF- κ B effected by UV exposure. HeLa cells were transfected with a NF- κ B-dependent reporter
25 gene and exposed to UV-C 24 hours after transfection. Pretreatment with Astaxanthin was done 3 hours prior to irradiation, where indicated. Cells were harvested and luciferase activity was measured 14-18 hours following exposure. The ratio between Firefly and Renilla luciferase activity is represented as fold activation over the activity in unstimulated control cells. The standard error is based on two identical experiments.

Experiments

The experiments were conducted in order to show that astaxanthin inhibits the activation of NF- κ B effected by UV exposure and thus the expression of inflammatory cytokines and chemokines.

MATERIALS AND METHODS

Cell culture

The human fibroblast cell line Hela Tet/off was cultured in MEM alpha medium supplemented with 10% fetal calf serum, penicillin and streptomycin (Gibco Grand Island, NY). The day before transfection, cells were plated on 6 cm dishes in a medium containing 2.5% fetal calf serum. On the day of transfection, the cell culture medium was changed to fresh medium (2.5% fetal calf serum).

Preparation of Astaxanthin

A stock solution of Astaxanthin was prepared by dissolving synthetic Astaxanthin (Sigma) in 99.6% ethanol. The concentration of Astaxanthin was measured by spectrophotometry, and the absorbance maximum at 474-479 nm was used to calculate the concentration, according to the formula: $Abs_{max}/210$.

Plasmids

The 6 κ B-Luc plasmid contains a firefly luciferase reporter gene driven by 6 NF- κ B binding sites cloned upstream of a TK promoter (Meyer et al. 1993). The pRL-TK plasmid contains a Renilla luciferase reporter gene driven by the TK promoter and is used as a control for transfection efficiency (Promega).

Transfection, UV irradiation and reporter gene analysis

Plasmid DNA (1 μ g of the 6 κ B-Luc reporter gene plasmid and 200 ng of pRL-TK control plasmid) was added to a mixture of 4 μ l fuGENE 6 transfection reagent (Boeringer Ingelheim, Germany) and 196 μ l serum free medium and incubated for 15 min at room temperature, according to the manufacturers protocol. DNA/fuGENE 6 was added to HeLa cells at 50% confluency and the cells were left in a 37°C incubator. 24 hours after transfection, the medium was removed and cells were exposed to UV. For this, a Stratalinker 1800 (Stratagene) emitting a wavelength of 254 nm was used and 10 J/m² was applied. The same medium was then added back to the cells, and the cells were incubated at 37°C. Where indicated, cells were pretreated with Astaxanthin for 3 hours, by adding various

concentrations of Astaxanthin to the medium. After addition, the Astaxanthin was left in the medium throughout the experiment.

Extracts were prepared 14-18 hours following exposure, and luciferase assays were performed using components of the luciferase assay system (Promega).

5 Results

To analyse whether Astaxanthin has any effect on the activation of NF- κ B we performed transient transfection experiments in HeLa cells. NF- κ B has been shown to be responsive to UV-irradiation (reviewed in Pitette *et al.*, 1997). Similarly, we demonstrate that exposure of the cells to UV-C induces expression of a transfected NF- κ B-dependent reporter gene (Fig. 1). Moreover, pretreatment of the cells with Astaxanthin at the highest concentration repressed this activation almost 3-fold. These data indicate that Astaxanthin has an inhibitory effect on NF- κ B, DNA binding activity of NF- κ B and/or translocation of the NF- κ B across the nuclear membrane by interfering with at the signalling components in the NF- κ B activation pathway.

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CLAIMS

1. Method of inhibiting the expression of inflammatory cytokines and chemokines in an animal or man, which comprises administration to said animal or man of at least one type of xanthophyll in an amount inhibiting the expression of inflammatory cytokines and chemokines in said animal or man.
2. Method according to claim 1, wherein the type of xanthophyll is astaxanthin.
3. Method according to claim 2, wherein the astaxanthin is selected from the group consisting of astaxanthin from a natural source, synthetic astaxanthin and mixtures thereof.
4. Method according to claim 3, wherein the natural source is a culture of the alga *Haematococcus sp.*
5. Use of at least one type of xanthophyll for the preparation of a medicament for the prophylactic and/or therapeutic inhibition of the expression of inflammatory cytokines and chemokines in an animal or man.
6. Use according to claim 5, wherein the type of xanthophyll is astaxanthin.
7. Use according to claim 6, wherein the astaxanthin is selected from the group consisting of astaxanthin from a natural source, synthetic astaxanthin and mixtures thereof.
8. Use according to claim 7, wherein the natural source is a culture of the alga *Haematococcus sp.*
9. Commercial package containing a medicament comprising at least one type of xanthophyll and written and/or data carrier instructions for administration to an animal or man of the medicament for the prophylactic and/or therapeutic inhibition of the expression of inflammatory cytokines and chemokines.
10. Commercial package according to claim 9, wherein the type of xanthophyll is astaxanthin.
11. Commercial package according to claim 10, wherein the astaxanthin is selected from the group consisting of astaxanthin from a natural source, synthetic astaxanthin and mixtures thereof.
12. Commercial package according to claim 10, wherein the natural source is a culture of the alga *Haematococcus sp.*
13. Commercial package according to any one of claims 9 - 12, which additionally contains a water soluble antioxidant and/or a fat soluble antioxidant other than the xanthophyll.

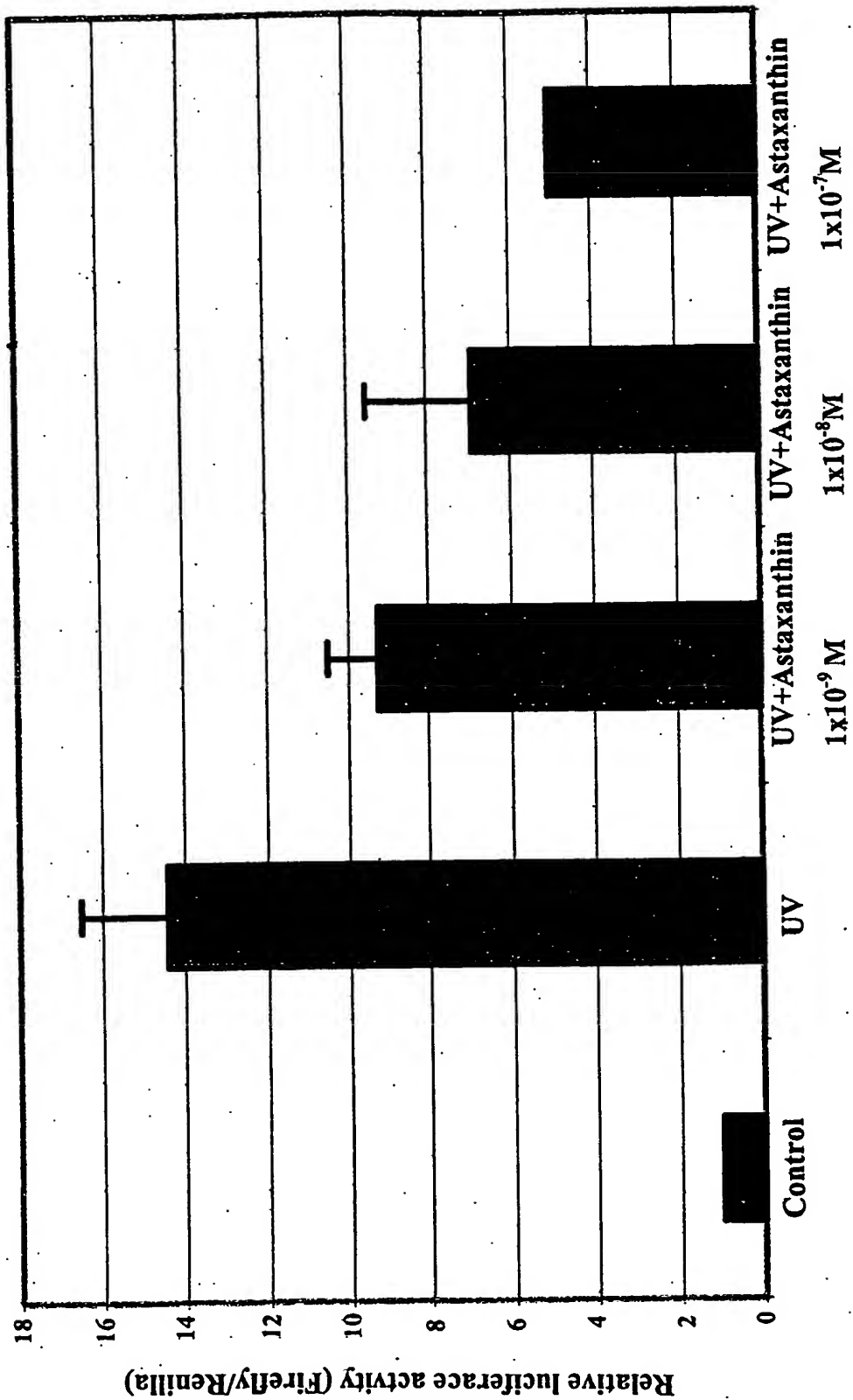


Fig. 1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 01/00600

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: A61K 31/12, C07C 403/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: A61K, C07C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9837874 A1 (ASTACAROTENE AB), 3 Sept 1998 (03.09.98), page 6, line 1 - line 30 --	5-13
X	Physiol. Chem. Phys. & Med. NMR., Volume 22, 1990, Michi Kurashige et al, "Inhibition of Oxidative Injury of Biological Membranes by Astaxanthin", page 27 - page 38, pages 35-37, 30 --	5-6
X	STN International, file CAPLUS, CAPLUS accession no. 1986:85415, document no. 104:85415, Popova, N. V. et al: "Carotenoids of the fruit of Capsicum annum"; & Farm. Zh. (Kiev) (1985), (6), 50-4, lines 13-26 --	5-6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"I" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

8 August 2001

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 01/00600

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9845241 A2 (HENKEL CORPORATION), 15 October 1998 (15.10.98), page 2, line 16; page 3, line 11; page 15, line 5 - line 10 --	5-6
X	US 5527533 A (MARK O. TSO ET AL), 18 June 1996 (18.06.96), column 6, line 5 - line 36; column 8, line 40 - line 45 --	5-13
X	Patent Abstracts of Japan, abstract of JP 7-99924 A (nippon suisan kaisha ltd), 18 April 1995 (18.04.95), claims 1-8 --	5-13
X	Patent Abstracts of Japan, abstract of JP 7-300421 A (Itano Reitou KK), 14 November 1995 (14.11.95), claims 1-7 --	5-13
A	WO 9911251 A1 (ASTACAROTENE AB), 11 March 1999 (11.03.99), page 1 - page 9 --	5-13
A	EP 0770385 A1 (SUNTORY LIMITED), 2 May 1997 (02.05.97), page 1, line 32 - line 46 --	5-13
A	US 5886053 A (WOLFGANG SCHMUTZLER ET AL), 23 March 1999 (23.03.99), column 1, line 25 - line 52 -- -----	5-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE01/00600

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: **1-4**
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see next sheet

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE01/00600

Claims 1-4 relate to methods of treatment of the human or animal body by surgery or by therapy/ diagnostic methods practised on the human or animal body/Rule 39.1.(iv). Nevertheless, a search has been executed for these claims. The search has been based on the alleged effects of the compounds/compositions.

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/07/01

International application No.

PCT/SE 01/00600

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
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